Immune Status of Autochthonous and Adoptively Protected Mice toward Spontaneous and Chemically Induced Tumors

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SUMMARY

The immune reactivity of mice toward their own primary tumors was evaluated immediately after surgical excision of the tumor, with or without procedures to establish adoptive tumor immunity. Methylcholanthrene (MCA)-induced sarcomas and "spontaneous" mammary carcinomas of C3H/He mice were used. Autografts of either of these tumors grew better than isografts into normal mice. Attempts to transfer this conditioned status from primary hosts bearing MCA tumors to syngeneic recipients were unsuccessful. Neither with lymphoid cells nor with serum could we induce such state in recipient mice in which latency and growth of primary and transplanted, MCA-induced tumors was measured. On the other hand, attempts to transfer specific immunity from actively immunized mice to syngeneic recipients were successful. When mice bearing primary or transplanted tumors were lethally irradiated and repopulated with lymphoid cells from normal or tumor-immunized donors, tumor growth was markedly inhibited. This treatment was effective for MCA-induced sarcomas both in inbred and random-bred mice, but not for spontaneous mammary carcinomas. Procedures involving repopulation of tumor-bearing mice with lymphoid cells from normal or immunized mice seem to correct a decreased level of reactivity of the animals toward their own tumors.

INTRODUCTION

Some human tumors have been shown to possess specific antigens (7) similar to those induced in laboratory animals by chemicals (6). The possibility of applying some forms of immunotherapy to human cancer encourages the testing of methods, in experimental models, which may be therapeutically successful in humans (1, 3, 9).

We have examined the ability of mice to reject 2 kinds of autochthonous tumors: mammary carcinomas arising spontaneously in C3H/He mice and sarcomas induced in the mesenchymal tissues of several mouse strains with 3-MCA. These experiments showed that the level of reactivity of the animals toward their own tumors was lower than that of normal syngeneic mice. On this basis, we have evaluated the effect of various treatments on tumor development, attempting to restore the capacity of the host to reject its tumor. By applying X-irradiation and injecting lymphoid cells i.v., we have studied the effect of lymphoid grafts from various donors, either tumor-bearing, normal, or immune, on the formation, growth, and grafting of primary tumors.

MATERIALS AND METHODS

Mice. Inbred mice of the strains C3H/He, C3Hf/He, DBA/2, BALB/c × DBA/2 F1 and C57BL/6 × BALB/c F1, and random-bred mice of the ICR albino strain were used. Skin and tumors grafted within any of the inbred strains are not rejected; grafts within the ICR albino strain are rejected.

Tumor Induction. Multiparous C3H/He females develop approximately 100% spontaneous mammary tumors (mostly carcinomas) in the 1st year of life. They are infected with the mammary tumor virus which is transmitted through the milk. In the same period, tumor incidence in the C3Hf strain is insignificant. These animals are syngeneic with the C3H/He but do not transmit the mammary tumor virus through the milk and can develop antibodies to it. Sarcomas were induced by s.c. implantation of paraffin pellets containing 0.192 or 0.320 mg MCA in the right hind limb.

Tumor Grafts. Mammary tumor cell suspensions were prepared by shaking finely minced tumor tissue in 0.9% NaCl solution and letting them settle for 3 or 4 min. Cell concentration was adjusted in the supernatant for inocula of 0.1 ml containing 5 to 100 × 103 trypan blue-unstained cells. Sarcomas were cut with a scalpel into cubes of 1 ± 0.2 mm which were implanted s.c. with a trocar. In some experiments, the sarcomas were finely minced with scissors and, after 20 min of digestion in 0.25% Pronase, the cells were washed in 0.9% NaCl solution, adjusted to a desired concentration with 100 units of penicillin and 100 units of streptomycin per ml and injected s.c.

X-irradiation. Whole-body X-irradiation (800 to 850 R) was administered under the following conditions to the mice: 190 kV, 20 ma, 0.5 mm Cu, and 1.0 mm Al filtration, at a target distance of 50 cm, giving approximately 38 R/min. One hundred % of the animals died within 40 days of receiving 800 R, unless they were injected with lymphoid cells.

Lymphoid Cell Injections. Cell suspensions were prepared by pressing the spleens and axillary, cervical, inguinal, and mesenteric lymph nodes through a stainless steel screen into Hanks' balanced salt solution containing 100 units of penicillin and 100 units of streptomycin per ml. Cell suspensions were filtered with a stainless steel screen into Hanks' balanced salt solution containing 100 units of penicillin and 100 units of streptomycin per ml.
and 100 µg of streptomycin per ml. After the suspension was decanted for 5 to 10 min, the supernatant was adjusted for i.v. inocula of 0.3 to 0.5 ml containing 50 to 100 X 10⁶ cells. Ninety % of the lethally irradiated mice which received these cell doses survived for at least 100 days. This procedure is referred to as “repopulation.”

Evaluation of Tumor Growth and Host Survival. The survival of the mice and the presence and size of tumors at the site of cell or carcinogen implantation were recorded weekly. The average diameter of each tumor was calculated from 3 different measurements to the nearest mm, each being made at planes perpendicular to each other. The incidence of primary tumors was scored when they attained 5 mm of average diameter. When tumor growth was compared in paired series of mice, each tumor in the experimental series was classified as growing either better, the same, or worse than its respective control. When differences were less than 0.5 mm, the sizes were considered the same. The significance of cumulative results from several pairs was calculated with the Sign test. For latent periods, “takes” and sizes of tumor grafts, the Mann-Whitney U test was applied.

Other procedures are individually described in each experiment.

RESULTS

Comparison of Autografts versus Cross-isografts of Tumors after Excision from the Primary Host

For evaluation of the ability of the primary host to reject or allow the growth of its own mammary tumor or MCA-induced sarcoma, pairs of C3H/He or C57BL/6 X BALB/c F₁ female mice with primary tumors of less than 10-mm diameter were matched for having growths of equivalent size. Tumors were excised broadly under Nembutal anesthesia by amputation of the whole limb in the case of the sarcomas or of the whole tumor-bearing mammary fat pad in the case of the mammary tumors. Within 1 hr, tumors were reinoculated into the same animals. Each mouse received its own tumor in the right flank and that of its mate in the left flank. At the same time a 3rd, nontreated syngeneic mouse (which for the mammary carcinomas was a multiparous C3H/He female) was sham-operated, added to the pair, and received in each flank a graft of each of the 2 tumors. Cell suspensions were used in the case of mammary carcinomas; measured fragments of equal size were used in the case of sarcomas.

Autografts grew better than isografts into normal, sham-operated mice [p = 0.01 for mammary tumors, p < 0.006 for MCA-induced sarcomas (Chart 1)], but not better than isografts into animals with recently excised tumors [p = 0.16 for mammary tumors, p > 0.1 for MCA-induced sarcomas (Chart 2)].

Attempts to Transfer the Conditioned Status of the Primary Host toward Its Own Tumor with Lymphoid Cells or Serum

Three procedures were used to test whether the better growth of tumors in the host of origin might be due to deficient cellular immunity or to the presence of blocking antibodies. In the 1st procedure, mice repopulated with lymphoid cells from primary tumor-bearing hosts were challenged with the respective tumors. In the 2nd procedure, mice similarly repopulated were challenged with the carcinogen MCA. In the 3rd procedure, the recipients of the primary tumor grafts were treated with serum from the corresponding tumor donors.

Growth of Tumor Isografts in Mice Repopulated with Lymphoid Cells from the Primary Tumor-bearing Hosts. These experiments were performed in DBA/2 mice and the repopulation procedure included s.c. thymus implantation besides the i.v. inoculation of lymphoid cells. We could not detect facilitation of the growth of inocula of either 10⁵ tumor cells or of small tumor fragments in animals repopulated with lymphoid cells from the respective primary tumor hosts. Some inhibition of growth (p = 0.029) was instead observed, suggesting that immunity rather than tolerance had probably been transferred (Table 1).

A control procedure, incorporated in the same animals of the mentioned experiment, showed that the method used
Adoptive Antitumor Immunity

Effect of Adoptive Immunity on Grafted Tumors. Investigations were made to see whether C57BL/6 X BALB/c F1 female mice, lethally irradiated and repopulated with lymphoid cells from a syngeneic mouse specifically immunized against a tumor, would adopt this immunity. The donors were immunized by implantation of tumor fragments and excision of the resulting tumors. Lymphoid cells were collected 1 to 8 weeks after excision and injected i.v. (5 X 10^6 cells) into lethally irradiated recipients. These recipients were challenged with 10^6 cells of the same sarcoma used to immunize the donors. Tumor rejections were significantly higher than in mice repopulated with lymphoid cells from normal donors. This result was obtained either when the lymphoid cells were inoculated 14 days before the tumor [prevention of tumors (Chart 3)] or 1 day afterwards (Chart 4), or 7 days afterwards [treatment of tumors (Chart 5)].

Treatment of Autochthonous Tumors by Adoptive Immunization. Since the forementioned procedures inhibited the growth of sarcoma grafts implanted previously, tests were made to see whether primary sarcomas would be inhibited by lymphoid grafts from specifically immunized mice. Groups of C57BL/6 X BALB/c F1 female mice bearing primary sarcomas were treated in the following way:

- **Group 1.** The tumor-bearing limb was surgically amputated and a cube of tumor tissue measuring 1 cu mm was autografted s.c. to the back of the same animal.
- **Group 2.** Amputation and autograft as in Group 1. Seven days later, the animals were irradiated with 800 R and received 50 to 100 X 10^6 normal syngeneic lymphoid cells.
- **Group 3.** Amputation and autograft as in Group 1. Seven days later, the animals were irradiated with 800 R and received 50 to 100 X 10^6 normal syngeneic lymphoid cells.

### Table 1

Comparison of tumor growth and BALB/c allograft survival between mice repopulated with lymphoid cells from primary tumor-bearing donors tolerant to BALB/c and mice repopulated with normal syngeneic lymphoid cells (DBA/2 strain)

<table>
<thead>
<tr>
<th></th>
<th>Better</th>
<th>Same</th>
<th>Worse</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allograft survival</td>
<td>7/8</td>
<td>1/8</td>
<td>0/8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tumor growth</td>
<td>11/36</td>
<td>2/36</td>
<td>23/36</td>
<td>0.029</td>
</tr>
</tbody>
</table>

* Average rejection time was 15 days in controls and >40 days in mice repopulated with lymphoid cells from tolerant donors.

(which failed to transfer "tolerance" to the tumor) was indeed capable of transferring allograft tolerance. The mice that provided the primary tumors had been previously made tolerant to BALB/c tissues by neonatal inoculation of BALB/c spleen cells and had proven to tolerate BALB/c skin in every case. This allograft tolerance, but not the "tumor tolerance," was transferred by the lymphoid cells to the repopulated recipients, as attested by successful grafts of BALB/c skin in most cases (Table 1).

Latent Period of Tumor Induction in Mice Repopulated with Lymphoid Cells from the Primary Tumor-bearing Hosts. C57BL/6 X BALB/c F1 mice were implanted with a 0.192-mg MCA pellet and after 12 days they received 800 R and 5 X 10^6 lymphoid cells i.v. from either normal syngeneic donors (control group) or donors which had had MCA implants for more than 80 days and in which tumors were already growing. Nine control and 14 experimental mice could be followed for 7 months and the latency of tumors was recorded. Both groups developed 100% tumors and not even slight differences could be detected in the latent periods. A confirmatory experiment in which the repopulation was performed 60 days after carcinogen implant was done with 16 experimental and 15 control mice. Again no differences in the latency were found. Additionally, the latent period was the same in mice repopulated with their own spleen cells as in those repopulated with spleen cells from other, MCA-treated mice.

Growth of Tumor Isografts in Mice Treated with Serum from the Primary Tumor-bearing Hosts. To test whether the better growth of tumors in primary than in syngeneic hosts might be due to blocking antibody present in the primary host, serum from BALB/c X DBA/2 F1 female mice bearing autochthonous, MCA-induced sarcomas was collected on repeated occasions during the period of tumor formation and growth, diluted 1:3 in 0.9% NaCl solution, and stored frozen in individual vials for each animal. When the primary tumors attained approximately 20 mm of average diameter, cell suspensions were prepared and incubated with the serum from the same donor for 45 min at room temperature. The incubation mixture was then injected into syngeneic recipient mice at doses of 4 X 10^6 to 4 X 10^6 cells in 0.1 ml of serum. On Days 2 and 4 after inoculation, the recipients were treated again by injection i.p. or s.c. at the tumor site with 0.1 ml of serum. Control animals were simultaneously inoculated and treated with normal serum. In 10 separate experiments involving 10 different primary tumors and a total of 143 experimental and 144 control recipient mice, we could not transfer the conditioned state of the primary host to the recipients of the 1st tumor isograft by means of the serum. This was shown by an equal tumor growth in both experimental and control groups in every case.

Chart 3. Growth of a MCA-induced sarcoma in mice repopulated 14 days before tumor inoculum with syngeneic spleen and lymph node cells from immunized mice.
50 to $100 \times 10^6$ syngeneic lymphoid cells from mice immunized against the tumor being treated by s.c. inoculations of tumor tissue irradiated with 14,500 R.

The autograft was measured weekly. Lethal irradiation and repopulation with normal or immunized lymphoid cells had a therapeutic effect on the primary autografted sarcomas (Chart 6). The effect was evident when comparison was made between the treated groups (Groups 2 and 3) and the nontreated Group 1. However, when only the effects of repopulation with normal versus immunized lymphoid cells were compared (Group 2 versus 3), the difference was statistically significant only in the 1st 2 weeks and then disappeared. In 2 experiments, the effects of treatment were also reflected in significant increases of the percentage of mice surviving (Table 2).

DISCUSSION

A decreased resistance to their own tumors was observed in mice bearing either primary MCA sarcomas or spontaneous
Adoptive Antitumor Immunity

Table 2
Survival of mice with autografts or isografts of MCA-induced sarcomas treated with lethal irradiation and lymphoid cell infusions

<table>
<thead>
<tr>
<th>Experiment and strain</th>
<th>Starting no. of mice</th>
<th>Treatment</th>
<th>% survivors at 30 days</th>
<th>% survivors at 60 days</th>
<th>% survivors at 100 days</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Graft prevention C57BL/6 x BALB/c F1</td>
<td>18</td>
<td>Graft</td>
<td>100</td>
<td>67</td>
<td>22</td>
<td>0.2185</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>800 R, NLC, graft</td>
<td>72</td>
<td>61</td>
<td>39</td>
<td>0.6617</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>800 R, ILC, graft</td>
<td>88</td>
<td>82</td>
<td>44</td>
<td>0.0001</td>
</tr>
<tr>
<td>Graft treatment C57BL/6 x BALB/c F1</td>
<td>23</td>
<td>Graft, 800 R, NLC</td>
<td>83</td>
<td>43</td>
<td>17</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>Graft, 800 R, ILC</td>
<td>92</td>
<td>72</td>
<td>73</td>
<td>0.5742</td>
</tr>
<tr>
<td>Autograft treatment C57BL/6 x BALB/c F1</td>
<td>28</td>
<td>Autograft, 800 R, NLC</td>
<td>100</td>
<td>78</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>Autograft, 800 R, ILC</td>
<td>100</td>
<td>59</td>
<td>41</td>
<td></td>
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</tbody>
</table>

* Results of similar experiments are pooled.

b NLC, normal lymphoid cells injected i.v.; ILC, lymphoid cells from specifically immunized mice given i.v. injection.

mammary carcinomas. This was detected when the growth of tumor autografts was compared to that of isografts into normal animals. The conditioned status of the primary hosts toward their own MCA sarcomas could not be transferred from tumor-bearing to normal syngeneic mice by means of lymphoid cells or serum. When, conversely, lymphoid cells were transferred from the normal to the tumor-bearing mice, resistance to the autochthonous tumors was increased. Furthermore, repopulation with lymphoid cells from specifically immunized mice was successfully used to prevent the growth of autografted MCA sarcomas. This procedure has no detectable effect against spontaneous mammary tumors.

Our results with cross-tumor grafts between primary hosts partially confirm those of Stjernswärd (12) who, in the same type of experiment showed that primary tumors grow better when autografted to the primary host than when grafted to normal syngeneic hosts. This has been extended to spontaneous mammary carcinomas, which are different in their histology and pathogenesis and are much less immunogenic (4). On the other hand, Stjernswärd's observation that the primary host is more susceptible to implants of its own tumor than to similar syngeneic tumors was not confirmed. However, he probably detected a real but subtle effect, demonstrable only with threshold cell doses but not with the uniform tumor fragments of our experiments. A possible reason for this effect might be the existence of residual heterozygosity in inbred mice.

That immunosuppression by the carcinogen is a reason for the better growth of tumor autografts has been conclusively shown by Stjernswärd for MCA-induced sarcomas (12). However, this may not be the only reason, for we have demonstrated the same effect with spontaneous mammary tumors, in which no chemical carcinogen seems to be involved and which are, by and large, nonimmunogenic for syngeneic hosts tolerant to the mammary tumor virus.

To see whether enhancing antibody might be the reason for the better growth of autografts than isografts of MCA-induced sarcomas, we have repeatedly attempted to transfer this effect by means of the serum, from the primary to syngeneic hosts. Our failure to do so cannot rule out an effect of blocking antibody, since its demonstration may depend on a more adequate procedure (5, 13).

The success in transferring adoptive immunity to MCA-induced sarcomas confirms previous observations by Delorme et al. (3) and others (1, 11, 15). We have, in addition, applied lethal irradiation before the lymphoid cell infusions. In theory, this should add 2 new effects: that of irradiating the tumor with a dose of X-rays higher than the tolerated whole-body doses and that of totally repopulating the lymphoid system with the infused cells, instead of just adding these to the circulation. The production of antibody by infused cells has indeed been shown to be stronger after lethal irradiation.

Chart 7. Treatment of spontaneous C3H mammary tumors with lymphoid cells (L. C.) from C3Hf immunized mice.

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irradiation (8, 10). The reasons for this increase are not clear, but it has been attributed to a proliferating stimulus on the infused immune cells by the depleted lymphoid organs. Our treatment, however, did not seem to improve remarkably the results previously obtained without irradiation (1, 3, 11) as we had expected. Although the therapeutic effect was clear in the sense of delaying or arresting tumor growth, in no case could we record the regression of an established tumor.

The inhibition of tumor growth induced by normal lymphoid cells was in several experiments as strong as that induced by cells from specifically immunized mice. This effect has been attributed to a partial restoration of the lymphoid system of the host, exhausted by the growing tumor, by new, immunologically competent cells (2, 11). Furthermore, in our experiments, when the host had been supporting tumor growth for a period before treatment, the action of normal cells seemed to be most marked.

It can be concluded from these observations and from the experiments on cross-tumor grafts that antigenic tumors grow in part at the expense of a partially exhausted immune response and that the therapeutic action of infused lymphoid cells is mainly restorative. The failure of demonstrating these effects in parallel experiments with spontaneous mammary tumors further emphasizes that they may only be relevant for tumors which are highly antigenic.

ACKNOWLEDGMENTS

The technical assistance of Mr. Alejandro Mayer, Miss JoAnn Gates, and Miss Patricia Dennis is gratefully acknowledged.

ADDENDUM

When the infusion of lymphoid cells was not preceded by X-irradiation, the therapeutic effect on MCA sarcomas or mammary tumors was still detectable, but to a lesser degree than when both procedures were applied together.

REFERENCES


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